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The presence of trace components significantly broadens the molecular response of *Aspergillus niger* to guar gum

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ABSTRACT

Guar gum consists mainly of galactomannan, and constitutes the endosperm of guar seeds that acts as a reserve polysaccharide for germination. Due to its molecular structure and physical properties, this biopolymer has been considered as one of the most important and widely used gums in industry. However, for many of these applications this (hemi-)cellulosic structure needs to be modified or (partially) depolymerized in order to customize and improve its physicochemical properties. In this study, transcriptome, exoproteome and enzyme activity analyses were employed to decipher the complete enzymatic arsenal for guar gum depolymerization by *Aspergillus niger*. This multi-omic analysis revealed a set of 46 genes encoding carbohydrate-active enzymes (CAZymes) responding to the presence of guar gum, including CAZymes not only with preferred activity towards galactomannan, but also towards (arabino-)xylan, cellulose, starch and pectin, likely due to trace components in guar gum. This demonstrates that the purity of substrates has a strong effect on the resulting enzyme mixture produced by *A. niger* and probably by other fungi as well, which has significant implications for the commercial production of fungal enzyme cocktails.

Keywords: Guar gum; *Aspergillus niger*; CAZymes; Plant biomass degradation; Transcriptome; Exoproteome

Introduction

Guar gum is an abundant biopolymer obtained from *Cyamopsis tetragonolobus* seed endosperm, used by the plant as nutrient reserve [1]. It mainly consists of a linear backbone chain of β-1,4-linked mannose units with randomly attached α-1,6-linked galactose units, chemically known as galactomannan [2]. It is therefore similar in structure to galacto(gluco)mannans that constitute one of the major components of softwood (coniferous trees) hemicellulose structure of lignocellulosic biomass and agricultural wastes [3]. The intrinsic properties and structure of this low cost material make it a target in many industrial applications such as food, pharmaceutical, cosmetic, textile, paper, and mineral industries [1,4]. However, the main obstacle to a more widespread utilization of this complex polysaccharide is the need to optimize and customize its depolymerization and modify its side-chain structure to obtain desired properties. Generally, gums are depolymerized by physico-chemical treatments, which often require long processing time and have a low degree of selectivity, and sometimes also change the structural integrity of the gum [1,4,5]. These disadvantages can be overcome using enzymatic hydrolysis [6].

Research into the industrial applications of guar gum has expanded significantly in recent years, but there is limited knowledge relating its enzymatic hydrolysis to the design of suitable enzyme mixtures that satisfy all the requirements of specific applications [4–6]. Most enzymes required to fully deconstruct plant biomass polymers are catalogued in the carbohydrate-active enzyme databases (CAZy) (http://www.cazy.org/), which can be classified into families of glycoside hydrolases (GHs), glycosyltransferases (GTs), carbohydrate esterases (CEs), polysaccharide lyases (PLs), and auxiliary activities (AAs) [7].

The hydrolysis of galactomannan containing polysaccharides requires the action of several enzymes such as β-mannanases, β-mannosidases, α-galactosidases, and acetyl-mannan esterases [8]. The β-mannanases cleave the mannan backbone randomly to liberate oligosaccharides of varying lengths, while β-mannosidases are exo-acting enzymes that release monomeric D-mannose residues from these oligosaccharides [2]. The α-galactosidases and acetyl-mannan esterases catalyze the release of galactose and acetyl groups from mannan, respectively [9,10] . Based on their amino acid sequence, fungal β-mannanases are classified to CAZy families GH5, GH26 and GH134, β-mannosidases to GH2, α-galactosidases to GH27 and GH36, and acetyl-mannan esterases to CE16 family [8,10]. In nature, many

fungi secrete a broad set of these CAZymes, but the filamentous fungus *Aspergillus niger* is among the most important microorganisms capable of producing a variety of CAZymes of industrial interest at low cost [11].

Since the deconstruction of complex polysaccharides by fungi occurs mainly extracellularly, combining transcriptome and exoproteome analyses provides a complementary approach to identify the enzyme mixtures fungi use for plant biomass deconstruction [12]. To our knowledge, no transcriptomic or exoproteomic study of fungi grown on guar gum has so far been conducted. In the present study, a multi-omic approach and enzyme assays were used to decipher the complete enzymatic arsenal for guar gum depolymerization by the highly efficient plant biomass decomposer *A. niger*. Surprisingly, we found that trace components of guar gum (Table 1) strongly affected the set of genes/enzymes expressed/produced.

Materials and methods

Strain and culture conditions

Aspergillus niger N402 was propagated and grown on complete medium (CM) or minimal medium (MM) [13]. Liquid cultures were grown in a rotary shaker at 250 rpm and 30°C. Transfer experiment was performed by growing the strain for 16 h in 1 L Erlenmeyer flasks that contained 250 mL CM supplemented with 2% D-fructose as carbon source. The mycelium was harvested by filtration and washed with MM without a carbon source. One gram (wet weight) of the mycelium was transferred to 250 mL Erlenmeyer flasks containing 50 mL MM supplemented with either 25 mM D-glucose (Sigma-Aldrich), 25 mM D-mannose (Sigma-Aldrich), 25 mM D-galactose (Sigma-Aldrich), 1% mannan (1,4-β-D-mannan from carob galactomannan, Megazyme) or 1% guar gum (Sigma-Aldrich) and incubated for 2 h. Mannan and guar gum cultures were also incubated for 8 h, 24 h and 48 h. All cultures were performed as biological duplicates. Mycelium was harvested by vacuum filtration, dried between towels and frozen in liquid nitrogen. The mycelium samples were stored at -80°C prior to RNA isolation.

RNA isolation and microarray preparation

RNA samples for microarray analysis were prepared using TRIzol (Invitrogen) according to the instructions of the supplier. RNA was purified using a Nucleospin RNA cleanup kit (Macherey-Nagel GmBh). The RNA integrity and concentration was verified with an Agilent 2100 Bioanalyzer using a RNA6000 LabChip kit (Agilent Technology). The RNA samples were amplified, labeled, hybridized, washed, and stained as described previously [14]. The microarray hybridization using the Affymetrix *A. niger* GeneChips (Affymetrix) was carried out by GenomeScan B.V. (The Netherlands, Leiden).

Analysis of transcriptome data

The microarray datasets retrieved from GenomeScan were processed under the Bioconductor software package version 2.8 (http://www.bioconductor.org/) together with house-made scripts performed in Perl (version 5.0) and Python (version 3.0). Utilizing the R statistical environment, the probe intensities were normalized for background using the robust multiarray average method [15] with only perfect match (PM) probes. The quantiles algorithm was used to perform normalization [16]. The normalized gene expression values were then analyzed with the statistical Cyber-T tool package (http://cybert.ics.uci.edu/) and edgeR to detect differentially expressed genes (DEGs). A false discovery rate (FDR) cut-off value of adjusted $p<0.05$ was set to assess statistical significance. Principal component analysis (PCA) using R programming language was performed to verify if the biological replicates were sufficiently similar. Only DEGs with FDR values lower than 0.05 and $\log_2$-fold changes greater than 1.0 were considered upregulated. The gene expression values were calculated by the median polish summary method [15]. The transcriptome data generated and used in this study has been deposited at the Gene Expression Omnibus (GEO) database with accession number: GSE119310 and GSE98572.

Gene expression clustering, visualization and annotation

Averaged values of all replicates were hierarchically clustered with the R package-"gplots" using the parameters of Euclidean distance and complete linkage method. Clusters were set manually based on the branch-length differences of the gene-tree. The putative functional annotations of the plant biomass targeted CAZy genes were based on previous studies with the *A. niger* strain CBS 513.88 [14,17], as well as on manual phylogenetic analysis.

Sample preparation for exoproteomic analysis

The extracellular proteins were harvested from the *A. niger* liquid cultures grown on guar gum or mannan after 24 h and 48 h of cultivation. The culture liquids were separated from the mycelia by filtering through Miracloth (Millipore), after which the collected liquid fractions were centrifuged at 4°C for 1h at 3500 × *g.* Proteins were extracted from the culture liquids by precipitation with two volumes of chilled 80% acetone (Sigma-Aldrich), 20% trichloroacetic acid (Sigma-Aldrich) and 20 mM DTT (Promega). The samples were incubated on ice for 1 h and centrifuged at 3200 × *g* for 30 min. The obtained pellet was re-dissolved in chilled 20 mM DTT and 80% acetone, and incubated at -20°C overnight. The mixture was centrifuged at 3200 × *g* for 30 min and the obtained pellet was dried and resuspended in 50 μl of 0.25% (w/v) Anionic Acid Labile Surfactant I (AALSI, ProgentaTM, Protea Biosciences) containing 20 mM ammonium bicarbonate (pH 7.8). Five micrograms of each fraction was treated with 10 mM DTT and 55 mM iodoacetamide and subjected to in-solution overnight trypsin digestion. The digestion solutions were desalted using C18 ziptips (Millipore, Billerica). The peptides were resuspended in 60 μL of 5% acetonitrile and 0.1% formic acid.

Five microliters of peptide digest was loaded onto 10 mm × 75 μm PicoFrit column (New Objective, Woburn, MA) packed with Jupiter 5 μm, 300 Å, C18 resin (Phenomemex, Torrance, CA) connected with a Velos LTQ-Orbitrap mass spectrometer (Thermo-Fisher). Peptide separation was done using a 90 min linear gradient generated by an Easy-LC II Nano-HPLC system (Thermo-Fisher). The acquired shotgun liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) data were searched against the *A. niger* strain NRRL3 protein databases downloaded from JGI MycoCosm (https://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html) corresponding to the best hit proteins. Protein quantification analysis was based on MS precursor ion signal determined by the Proteome Discoverer Quant 1.3 (Thermo-Fisher) software using the extracted ion chromatograms for the m/z values of the three of the most intense peptide assigned to each protein. These values were normalized using the values calculated for the spiked pre-digested Bovine Serum Albumin (Michrom, Auburn, CA) internal standard to obtain the Ion Profile (IP) of each protein.

Exoproteomic data analysis

The normalized protein areas were used as the measurement of abundance level in the exoproteome analysis. Normalized values for all substrates are included in the Supplementary Table S1. For each protein at each time point, two biological replicates were tested and averaged. Differential protein expression data was filtered by Student *t*-test with a *p*-value cutoff 0.05. Relative protein abundance was expressed as % of total protein (% TE) values using the individually determined IP values within the same sample, and visualized with ggplot2 package in the R environment.

Protein concentration measurements and enzyme activity assays

The total extracellular protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Extracellular α-galactosidase (AGL), β-glucosidase (BGL), β-galactosidase (LAC), β-mannosidase (MND), α-xylosidase (AXL), β-xylosidase (BXL), cellobiohydrolase (CBH), glucoamylase (GLA), α-rhamnosidase (RHA), and α-arabinofuranosidase (ABF) activities were assayed in a total volume of 100 µL using 10 µL of a 2 mM *p*-nitrophenol (pNP) linked substrate (Sigma-Aldrich) solution, 20 µL culture liquid sample, and 25 mM sodium acetate, pH 5. The samples were incubated at 30ºC for 30 min and the reactions were stopped by the addition of 100 µL 0.25 M Na_2CO_3, pH 5.0. These conditions are commonly used for assays in *A. niger*, while the 30 min incubation was determined based on tests with different time points. Absorbance was measured at 405 nm in a microtiter plate reader (Biorad Model 550). For the determination of specific activities, pNP concentrations of 1-10 mM were used. Endo-1,4-β-mannanase (MAN) activity was measured using Azo-carob galactomannan (S-ACGLM) (Megazyme), according to the manufacturer's instructions. One unit of enzymatic activity was defined as the amount of protein required to release one µmol of the corresponding product per minute, under the assay condition used. All enzyme activity assays were calculated from two biological replicate cultures and three technical replicate measurements.

Results and discussion

In this study, we aimed to gain a comprehensive insight into the transcriptome of *A. niger* during guar gum degradation (after 2, 8, 24 and 48 h) and compare this to the transcriptome on the main guar

gum components D-mannose, D-galactose and mannan, as well as D-glucose that was used as a control (all after 2 h incubation). In addition to transcriptomics, the exoproteomes from guar gum and mannan, after 24 and 48 h, were analyzed to facilitate the identification of extracellular CAZymes, especially those with known function in degradation of plant biomass.

Functional distribution of CAZymes in A. niger transcriptomes and exoproteomes

Expression of 173 genes encoding (putative) plant biomass degrading CAZymes was detected in the microarrays, which included 143 GHs, 14 CEs, 8 PLs and 8 AAs from 41 CAZy families (Fig. 1A, Table S1-1A). Overall distribution of genes according to their CAZy families showed a large variety of functions. Of these, 57, 24, 23, 21, 17, 14, 11, and 6 CAZy genes were putatively targeted towards pectin, cellulose, (arabino)xylan, diverse activities, galactomannan, starch, xyloglucan and inulin, respectively (Fig. 1A, Table S1-1A). The majority of identified transcripts (57), as well as the higher number of CAZy family genes (GH28 with 20 genes), were found within CAZy genes involved in pectin degradation. Moreover, 17 genes from 6 different families were directly connected to galactomannan degradation (Fig. 1A, Table S1-1A). Diverse activities included 21 CAZy transcripts, which can have activity towards multiple substrates or unknown functions.

In addition, LC-MS/MS identified a total of 153 proteins in the extracellular liquid from *A. niger* guar gum and mannan cultures, of which 136 proteins possessed putative secretion signal peptides (Table S2-2A). The absence of a signal peptide from the 17 proteins could be due to the fact that possibly not all cleavage motifs for proteins have been identified, or alternatively due to technical problems during sample processing (e.g. proteins without trypsin cleavage sites) [18]. However, we do not rule out the possibility that these proteins may be subject to a non-classical secretory mechanism or may be released after cell lysis [19]. From these 153 proteins, 69 belonged to CAZymes with known functions in plant biomass degradation (Fig. 1B, Table S2-2B). Their distribution over the CAZy families was 58 GHs, 7 CEs, 1 PLs and 3 AAs, which is similar to the distribution observed based on gene expression. A large number and diversity of GHs were found in the exoproteomes, targeting a broad range of polysaccharides. In total, 22 proteins were related to pectin, 13 to (arabino)xylan, eight to cellulose, seven to xyloglucan, seven to galactomannan, six to diverse activities, four to starch and two to inulin degradation (Fig. 1B, Table S2-2B).

Of the CAZy families predicted to encode galactomannanolytic activities, GH27 again was the largest CAZy class found within this functional classification. Guar gum, while being a galactomannan in structure, contains traces of other polymers, such as starch, cellulose, xyloglucan or pectin [1,5,20]. This diversity of compounds may lead to the release of a range of monomeric compounds (e.g. L-arabinose, D-xylose, D-galacturonic acid, L-rhamnose, D-glucose), even though present at low level, which likely explains the broad diversity of enzymatic functions that are induced during growth on plant biomass [17]. It has previously been shown that already at 1 mM concentrations, and probably even lower, sugars can act as inducers of gene expression in *A. niger* [21]. The monosaccharides released from these impurities have been demonstrated to activate several transcriptional activators involved in plant biomass degradation [22,23].

Transcriptomic analysis and identification of differentially expressed CAZy genes on guar gum

To investigate substrate and time-dependent variation of the expression of the CAZyme encoding genes, four time points (2, 8, 24, and 48 hours) from *A. niger* grown on guar gum were compared to D-glucose (Glc2h), D-mannose (Man2h), D-galactose (Gal2h) and mannan (Mnn2h) cultivations at an early time point (2 h) of induction. Hierarchical clustering of the differentially expressed CAZy genes from the different C-sources after 2 h of cultivation and over a 48 h time-course of guar gum depolymerization was performed to compare gene expression (Fig. 2). These CAZymes were divided into eight groups based on their putative substrate specificities: cellulose-specific, (arabino)xylan-specific, xyloglucan-specific, galactomannan-specific, starch-specific, pectin-specific, inulin-specific and not specific substrate.

For the *A. niger* transcriptome data from guar gum and related carbon sources at 2 h of induction, nine clusters with distinct expression patterns were identified (Fig. 2, Table S1). When the substrate-based differential expression was compared, the D-mannose transcriptome revealed that it was highly similar to that of D-glucose and did not result in upregulation of any mannanolytic genes of *A. niger* (Fig.2, Table S1-1B). In contrast, a clear relationship between guar gum and mannan was observed (Fig. 2A, Table S1-1B). Considering that mannobiose was recently demonstrated to be the inducer of mannanolytic genes in *Aspergillus oryzae* [3], our analysis shows that the mannan fraction in guar gum was largely responsible for its expression profile.

The genes commonly induced by all four C-sources were present in clusters C1, C4 and C5, and encode cellulolytic, pectinolytic, amylolytic and galactomannanolytic enzymes (Fig. 2A, Table S1-1B), confirming the results from previous studies where α- and β-glucosidases, α- and β-galactosidases, CBHs, xylanases, arabinanases, as well as genes encoding α-amylases and GLAs were detected during the utilization of D-glucose and D-galactose by *A. niger* [24,25]. In contrast, the three genes of cluster C2, encoding an α-amylase (*amyA*), an exo-1,3-galactanase (EXG) and an α-xylosidase (*axlA*), were highly expressed on all the tested substrates except mannan and D-mannose (Fig. 2A).

Clusters C3 and C6 contained 12 genes specifically induced on mannan and guar gum, including genes encoding an β-xylosidase (*gbgA*), three α-arabinofuranosidases (*abfA*, *abfB*, *abfC*), an β-glucosidase (*bgl4*), an α-galactosidase (*aglB*), an β-mannosidase (*mndA*), two β-galactosidases (*lacA*, *lacB*), an endoarabinanase (*abnA*), a putative acetylmannan esterase (AME) and an exo-1,3-galactanase (*exgA*) (Fig. 2A, Table S1-1B). Although there is no clear evidence of the participation of β-galactosidases in the hydrolysis of galactomannan, our results suggest that these enzymes play a key role during guar gum degradation. The mannan specific genes were found in cluster C7, which included two β-glucosidases, a cellobiohydrolase (*cbhB*), an α-galactosidase, an endomannanase (*manA*), a rhamnogalacturonan acetyl esterase (*rgaeB*), a rhamnogalacturonan lyase (*rglA*), an endoarabinanase, and a putative β-glycosidase (Fig. 2A, Table S1-1B). The ManR transcription factor that is activated by glucomannan has been shown to regulate expression of α-galactosidase, β-mannosidase, acetylmannan esterase, β-glucosidase and cellobiohydrolase genes in *Aspergillus oryzae* [3,26]. The similarities in the transcriptome data of mannan and guar gum, as well as between the genes in these clusters and those described in the *A. oryzae* study [3,26], suggest that ManR also has a major role in controlling gene expression on these two substrates in *A. niger*.

The 15 genes of cluster C8 were specifically induced on guar gum and encode a broader set of enzymes than those induced on mannan, and mannan and guar gum. These include (arabino)-xylanolytic, cellulolytic, pectinolytic, inulinolytic and galactomannan active enzymes, such as β-xylosidases (An02g00140, *xlnD*), arabinoxylan arabinofuranohydrolase (*axhA*), α-glucuronidase (*aguA*), ferulolyl esterase (*faeB*), β-glucosidase (*bglM*), α-galactosidase (*aglA*), β-galactosidase (*lacC*), invertases (*sucA*, *sucB*), exo-1,6-galactanase (An03g01050), α-amylase (*amyC*), and α-fucosidase (*afcC*) (Fig. 2A, Table S1-

1B). Expression of this broader set of functions on guar gum is likely the result of the impurities in guar gum (Table 1) that trigger the activation of several transcription factors. The expression of *xlnD* and *aguA* was shown to be controlled by the hemicellulolytic regulator XlnR [27], which responds to the presence of xylose [28], while *axhA* is under control of both XlnR [27] and AraR, which responds to the presence of L-arabinose [29]. This indicates the role of monosaccharides not originating from galactomannan in gene expression during growth on guar gum. Apparently, these inducing compounds persist over time, suggesting that they are either not preferential carbon sources, or that they are only slowly released from the oligomeric or polymeric structures they are compounds of. Finally, cluster C9 contained 99 genes that did not have a significant difference of expression in all the C-sources tested (Fig. 2A, Table S1-1B).

The time-dependent clusters during growth of *A. niger* on guar gum were well separated according to the studied time points. As shown in Fig. 2B and Table S1-1C in Supplementary data, expression of many plant biomass degradation related CAZy genes increased strongly from 2 h to 8 h and then reduced again at the later time points. This expression profile can be explained by the release of monosaccharides that act as inducers for several transcriptional activators as mentioned above. As these other inducing compounds are only present in very small amounts, it is not surprising that their influence disappears again at later time points, resulting in a narrower set of expressed genes.

Fifty-four CAZy genes from clusters C1 and C9, including activities against (arabino)xylan, cellulose, galactomannan, pectin and starch, were medium to highly expressed independent of the time point (Fig. 2B, Table S1-1C), with several of them being the most abundant CAZy genes expressed. In contrast, 72 genes grouped in C5 and C6 did not have a differential expression pattern during 48 h of guar gum induction (Fig. 2B, Table S1-C).

Eight 2 h specific genes were found in clusters C2 and C3, including an α-arabinofuranosidase (*abfA*), a β-xylosidase, a feruloyl esterase (*faeB*), a β-galactosidase (*lacB*), two α-galactosidases (*aglC*), a β-fructofuranosidase/invertase (*sucA*) and an α-amylase (*amyA*) (Fig. 2B, Table S1-1C). Several of these genes (*amyA*, *aglC*, *sucA*) have been shown to be expressed in the presence of low levels of D-glucose [24] or sucrose [30], suggesting that free sugars present in guar gum are likely responsible for this expression profile. As these would be quickly consumed by *A. niger*, this likely explains the reduction in their expression at later time points.

283 The 8 h time specific genes were found in clusters C4 and C8. A high number of those genes
284 encoded pectinolytic enzymes, such as *pmeB*, *pelA*, *xghA*, *pgaC*, *pgaE*, *pgaII*, *pgaX*, *pgxB*, *lacA*, *rhgA*,
285 *rglA*, *rgaeA* and *rgxC* (Fig. 2B, Table S1-1C). A similar gene expression pattern corresponding to enzymes
286 acting on the pectin backbone has previously been observed for *A. niger* in presence of D-galacturonic acid
287 and L-rhamnose during the initial degradation of pectin [31]. However, a low expression of exo-pectinolytic
288 genes after 8 h was observed, indicating that possibly the main pectin chains are metabolized first, which
289 would make the main chains of these polymers more accessible to endo-enzymes at later stages of fungal
290 growth. Apparently, guar gum contains small traces of pectin polysaccharides or polymer decorations that
291 induced the expression of exo-acting pectinolytic GHs mostly during early degradation. The (temporal)
292 diversity of expression of pectinolytic genes has previously been reported for a selected set of genes [31],
293 and has recently been confirmed in a global CAZy expression study [17] and two studies addressing the
294 molecular mechanism of pectinolytic regulation [32].
295 Cluster C7 mainly contained genes that were not or lowly expressed at 2h, (highly) induced at 8h
296 and remained expressed at 24 h and 48 h (Fig. 2B). The majority of these genes corresponded to (arabino)-
297 xylanolytic, cellulolytic, xyloglucanolytic and not specific functions, such as *axeA*, *xlnC*, *faeA*, *eglC*, *cbhA*,
298 *eglC* and lytic polysaccharide monooxygenases (LPMOs) (Fig 2B, Table S1-1C). The strong induction of
299 those genes was an unexpected result, since the trace contaminants would be expected to be metabolized
300 by the fungus in the first hours of degradation, and guar gum does not contain cellulose or high
301 concentrations of xylose. This observation could suggest that the late induction of those genes is
302 triggered even when no inducing sugar is present. Interestingly, all of these genes have been shown to be
303 regulated by the zinc finger transcription factor XlnR in *A. niger* [17,28,33]. A detailed analysis of an *A.*
304 *niger xlnR* deletion mutant revealed low levels of expression of a subset of genes coding for degradative
305 CAZymes induced by carbon starvation, which can scout for available carbon source [25]. This finding may
306 explain why the induction of xylanolytic and cellulolytic genes occurs without the presence of inducer sugars
307 in later stages of cultivation on guar gum. In contrast, the regulation of galactomannanolytic genes showed
308 time-dependent variation, reaching a maximum expression level at 8 h (Fig 2B, Table S1-1C). The *A. niger*
309 genome contains 12 candidate CAZymes related to the degradation of the main chain and side-chains of
310 galactomannan [34,35]. However, the presence of a single α-galactosidase, β-mannosidase and endo-

mannanase has been shown to be sufficient for efficient degradation of galactomannan from different carbon sources [2,8,9]. The early upregulation of the exo-acting galactomannanolytic genes could be related to the presence of oligosaccharides in guar gum that would allow a quick release of D-galactose and D-mannose from the substrate.

Comparative analysis of the upregulated CAZy transcripts from substrate and time-dependent variation

We generated Venn diagrams to compare upregulated genes from different substrates after 2 h of incubation on guar gum (GG2h) vs. 2 h of incubation on D-glucose (Glc2h), D-mannose (Man2h), D-galactose (Gal2h) and mannan (Mnn2h). Overall, six and two genes were specifically upregulated after 2 h on guar gum compared to D-glucose, and D-mannose, respectively (Fig. 3A, Table S1-1D). In addition, seven genes were upregulated on guar gum compared to D-glucose and D-mannose, seven compared to D-glucose, D-mannose and D-galactose, four compared to D-glucose, D-mannose and mannan, and one gene compared to D-glucose and mannan. A core set of 10 genes was upregulated on guar gum compared to all four other substrates, including three xylanolytic (*axhA*, *axlA* and *aguA*), two cellulolytic (An07g09760 and *bglM*), an inulinolytic (*sucA*), an amylolytic (*amyA*), and two pectinolytic (*rha* and *lacA*) genes. The absence of galactomannan specific genes in any of these comparisons is likely in part due to them being mainly expressed at later time points during growth on guar gum (see below). Also, most of the genes differentially expressed between guar gum and the other substrates after 2 h have been demonstrated to be under control of XlnR, AraR, AmyR and/or InuR [17,30].

A similar comparison was performed to identify the time dependent upregulated genes on guar gum, by comparing the 2 h data (GG2h) to that of 8 h (GG8h), 24 h (GG24h) and 48 h (GG48h), resulting in a total of 106 significantly differentially expressed genes (Fig. 3B, Table S1-1E). As expected from the analysis described above, the largest number of specifically differently expressed genes was observed for the comparison between 2 h and 8 h (34), while the number was much lower for the comparison with 24 h (4) and 48 h (5). Most of these genes have no known role in galactomannan degradation, suggesting that they are likely expressed in response to monosaccharides present in the substrate or released from the impurities at later time points. The exceptions were two AGLs and one AME (8 h) and one MND (24 h). In contrast, only 13 genes were upregulated at all later time points compared to 2 h, consisting of four

cellulolytic genes (*cbhA*, *cbhB*, *eglA*, e*glC*), two amylolytic genes (*aamA*, *amyC*), one xyloglucanolytic gene (*xeg*), one arabino-xylanolytic gene (*axeA*), two mannanolytic genes (*manA*, AME), and three genes with a not yet defined substrate (one multicopper oxidase encoding gene, *mcoB*, and two LPMO encoding genes). The expression pattern of *manA* suggests that significant degradation of galactomannan does not occur until 8 h of cultivation and supports the preferential hydrolysis of smaller oligomer by the exo-acting enzymes before the polymeric fraction of guar gum is hydrolyzed.

Exoproteome analysis of guar gum and mannan degradation by A. niger

The exoproteomes of *A. niger* grown on guar gum and mannan were analyzed to examine the extracellular enzymatic responses during the degradation of guar gum in the later stages of the fungal cultivations. A larger number of proteins, 59 and 27, was detected at 48 h compared with 48 and 18 proteins at 24 h of guar gum and mannan induction, respectively, suggesting accumulation of secreted enzymes in the culture medium over time (Fig. 4, Table S2-2B). However, it was evident that there was a notable difference between the abundance of transcripts and the extracellular proteins detected. These differences seem to be more related to different factors such as biological process of gene regulation during and after the transcription, mechanisms of induction and repression of sugar catabolism, changes in the environmental conditions (pH, temperature, and ionic strength), or even technical artifacts during sample processing [12,18,34]. Nevertheless, a good correlation between expression levels and LC-MS/MS identification of secreted proteins were noted.

The number of extracellular (arabino)xylanolytic proteins increased from 7 to 13 and 1 to 3 in guar gum and mannan, respectively (Fig. 4). Cellulolytic proteins increased from 6 to 8 and 3 to 4 in guar gum and mannan, respectively. The number of galactomannanolytic proteins was more similar between both substrates. In contrast, inulinolytic proteins were not detected in mannan cultures. The number of pectinolytic proteins remained constant (16) at 24 and 48 h of induction on guar gum, whereas a small number of proteins was found at both time points for mannan (from 4 to 6). Similarly, the number of xylanolytic proteins increased in guar gum, but only two proteins were detected in mannan. The amount of amylolytic proteins during later stages of degradation of guar gum and mannan was very similar.

It was noticed that despite the relatively low total abundance of pectin degrading proteins on both substrates, a highly secreted endoarabinanase (*abnC*) was detected only in the later stages of the mannan cultivations (Fig. 4). As expected, the relative abundances of the proteins within galactomannan functional category were similar during the cultivation in guar gum and mannan (Fig. 4). The most abundant proteins were found within this category, including an An02g11150 GH27 AGL, an An05g01320 GH5 MAN, an An11g06540 GH2 MND, and an An07g08940 CE16 AME (Fig. 4, Table S2-2C). Taken together, our results confirm the previous observations of their orthologs (*aglB*/AO090003001305, *manG*/AO090010000122, *ameA*/AO090005001552 and NCU08412) in the distantly related species *A. oryzae* [3] and *N. crassa* [36] as critical enzymes for mannan utilization. The combined results of the exoproteome and the transcriptome analysis confirmed that, in this experimental setting, mannan and guar gum induced a similar pattern of expression of genes encoding galactomannan specific enzymes and these enzymes were secreted.

The presence of a large variety of (arabino)xylan, inulin, pectin, xyloglucan degrading enzymes led us to hypothesize that only some of these enzymes may be important for guar gum (galactomannan) hydrolysis, while the others were induced by the presence of trace components present in the guar gum used in this study.

Although guar gum is extracted from the same plant species, its molecular composition can be influenced by the conditions and method of extraction used [4]. Previous physico-chemical analysis have shown that pure guar gum can contain up to 13% of impurities such as soluble sugars and traces of proteinaceous matter [4,20], which may originate from the remaining seed coat in the extracted guar gum. Our results suggest that this low amount of impurities acts as a potent inducer for the production of many hydrolases (even at low concentrations), suggesting that the purity of the substrate strongly affects the response of the fungus. Therefore, the difference in gene expression compared to the monosaccharides could be accounted to the activation of the hydrolytic enzymes targeted for the substrate impurities of guar gum.

Additionally, the transcriptome and exoproteome of *A. niger* were compared, resulting in a common set of 46 significantly upregulated CAZymes shared between them. This set contained 10 (arabino)-xylanolytic, 10 pectinolytic, seven galactomannanolytic, six cellulolytic, five xyloglucanolytic, four amylolytic and two inulinolytic genes/enzymes (Table 2). We also detected LPMOs and MCO-related enzymes after

8 h of induction on guar gum in the exoproteomic and transcriptomic samples, even though their corresponding genes were not highly expressed (Fig. 2, Table S1 and S2). Recently, Nekiunaite et al. [37] identified several oxidoreductases co-expressed with amylolytic hydrolases during the deconstruction of starch-containing substrates by *Aspergillus nidulans*. The role of these oxidoreductases could possibly be in the oxidative cleavage of linkages in guar gum to boost its hydrolysis by the hydrolytic depolymerizing enzymes [38,39].

Validation of the activities of the guar gum related CAZymes produced by A. niger

To estimate the potential of the upregulated CAZymes identified by transcriptomic and exoproteomic analyses, several enzyme activities were determined in samples from the cultures after 8 h, 24 h and 48 h: α-galactosidase (AGL), β-galactosidase (LAC), endomannanase (MAN), β-mannosidase (MND), α-xylosidase (AXL), β-xylosidase (BXL), β-glucosidase (BGL), cellobiohydrolase (CBH), glucoamylase (GLA), rhamnosidase (RHA) and arabinofuranosidase (ABF).

The results show that *A. niger* produced all the 11 tested activities during growth on guar gum and mannan (Fig. 5). At 8 h, most of the enzymes showed a reduced activity in comparison with 24 h. After 24 h, AGL, MAN, MND, BGL, CBH, GLA, and ABF activities increased substantially on both guar gum and mannan. Likewise, at 48 h cultivation on mannan, AGL and MND activities increased strongly compared with those detected on guar gum. However, at the same time point of analysis, the BGL, CBH, and GLA activities from guar gum treatments were higher than that of mannan. Interestingly, only the MAN activities of guar gum and mannan cultures showed similar levels of activity. The maximal galactomannanolytic activities were reached for AGL, MAN and MND after 48 h of incubation, which was also the case for cellulolytic and several xylanolytic activities (Fig. 5). In contrast, AXL, BXL and RHA activity were low compared to the other enzyme activities. This difference correlates with the weak expression of RHA in *A. niger* and the fact that this enzyme has a low activity [17,40]. The measured enzyme activities were generally in good agreement with the putative key CAZymes found in the *A. niger* transcriptomes as well as in the exoproteomes during cultivation on guar gum.

Conclusion

This is the first report with an integral approach to identify the key enzymes produced by *A. niger* during growth on guar gum. Interestingly, guar gum, consisting mainly of galactomannan, triggered the production of a rich and complex set of hydrolytic CAZymes with most pronounced activities towards (arabino)-xylan, cellulose, galactomannan, and pectin. This indicates that the small amounts of other mono-, oligo- and polysaccharides than galactomannan in guar gum have a major influence on gene expression and protein production and can strongly alter the composition of the enzyme mixture produced by this fungus. The implications of this for commercial production of enzyme cocktails are very high, as this indicates that variations in the impurities of feed stocks used for enzyme productions may significantly alter set of enzymes in the resulting cocktail and therefore its effectivity.

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555 **Table 1.** Composition analysis of guar gum.

Component	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Total sugar
Mol%	2.2	0.4	56.9	35.4	3.2	1.7	83.6

556

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Table 2. CAZymes related to biomass deconstruction that were significantly ($p<0.05$) upregulated in the *A. niger* N402 transcriptomes and exoproteomes from guar gum compared to mannan.

ID_CBS513.88	CAZy family	Enzyme code/gene	Function	Putative substrate	log2 fold change
An18g04810	GH28	RGX_*rgxC*	exo-rhamnogalacturonase	pectin	8.639498
An16g00540	GH95	AFC	α-fucosidase	xyloglucan	6.932792
An15g05370	GH28	PGA_*pgaII*	endopolygalacturonase	pectin	6.842149
An05g01320	GH5	MAN_*manA*	endomannanase	galactomannan	6.365687
An01g12150	GH35	LAC_*lacA*	β-galactosidase	galactomannan, pectin	5.866629
An14g02760	GH12	XEG_*eglA*	xyloglucan-active endoglucanase	xyloglucan	5.466683
An07g08940	CE16	AME	putative acetylmannan esterase	galactomannan, xylan	5.377879
An08g11070	GH32	SUC_*sucA/sucI*	invertase/β-fructofuranosidase	inulin	5.356922
An14g01790	GH27	AGL	α-galactosidase	galactomannan	4.930603
An15g02300	GH54	ABF_*abfB*	α-arabinofuranosidase	(arabino)-xylan, pectin	4.360588
An02g11150	GH27	AGL_*aglB*	α-galactosidase	galactomannan	4.291878
An09g01190	GH43	ABN_*abnA*	endoarabinanase	pectin	4.154161
An11g06540	GH2	MND_*mndA*	β-mannosidase	galactomannan	4.142109
An08g10780	GH43	BXL_*gbgA*	ß-xylosidase	(arabino)-xylan	3.956593
An01g00330	GH51	ABF_*abfA*	α-arabinofuranosidase	(arabino)-xylan, pectin	3.838550
An03g06740	GH28	PGX_*pgxB*	exopolygalacturonase	pectin	3.367877
An07g08950	GH5	EGL_*eglC*	endoglucanase	cellulose	3.062056
An04g09700	GH28	XGH_*xghA*	xylogalacturonase	pectin	3.046303
An08g01900	GH43	BXL	β-xylosidase	(arabino)-xylan	3.023174
An09g03300	GH31	AXL_*axlA/xylS*	α-xylosidase	xyloglucan	2.992657
An11g00200	GH3	BGL_*bglM*	β-glucosidase	cellulose	2.979334
An01g10350	GH35	LAC_*lacB*	β-galactosidase	galactomannan, pectin	2.939864
An07g09330	GH7	CBH_*cbhA*	cellobiohydrolase	cellulose	2.922187
An08g01710	GH51	ABF_*abfC*	α-arabinofuranosidase	(arabino)-xylan, pectin	2.915692
An16g02760	GH95	AFC_*afcC*	α-fucosidase	xyloglucan	2.753815
An06g00170	GH27	AGL_*aglA*	α-galactosidase	galactomannan	2.679696
An12g08280	GH32	INX_*inuE/inuI*	exo-inulinase	inulin	2.617590
An02g02540	CE16	AME	putative acetylmannan esterase	galactomannan, xylan	2.554184
An04g09690	CE8	PME_*pmeB*	pectin methylesterase	pectin	2.494095
An03g00940	GH10	XLN_*xlnC/xynA*	β-1,4-endoxylanase	(arabino)-xylan	2.436745
An03g01050	GH5	GLN	exo-1,6-galactanase	pectin	2.184780
An02g10550	GH43	ABN_*abnC*	endoarabinanase	pectin	2.120337
An04g06920	GH31	AGD_*agdA*	α-glucosidase	starch	2.095693
An11g03340	GH13	AMY_*aamA*	α-amylase	starch	2.014945
An12g10390	CE1	FAE_*faeB*	feruloyl esterase	(arabino)-xylan, pectin	1.895975
An03g06550	GH15	GLA_*glaA*	glucoamylase	starch	1.854618
An11g02100	GH1	BGL	β-glucosidase	cellulose	1.692845
An01g13660	AA1	MCO_*mcoB*	multicopper oxidase	not specific	1.599671

An03g00960	GH62	AXH_*axhA*	arabinoxylan arabinofuranohydrolase	(arabino)-xylan	1.565415
An01g09960	GH3	BXL_*xlnD/xynD*	β-xylosidase	(arabino)-xylan	1.534312
An01g11660	GH7	CBH_*cbhB*	cellobiohydrolase	cellulose	1.510050
An08g04630	GH131	EGL	endoglucanase	cellulose	1.469976
An01g01870	GH74	XEG_*eglC*	xyloglucan-active endoglucanase	xyloglucan	1.461051
An14g02670	AA9	LPMO	lytic polysaccharide monooxygenase	not specific	1.394944
An01g10930	GH31	AGD_*agdB*	α-glucosidase	starch	1.344306
An01g00780	GH11	XLN_*xlnB*	β-1,4-endoxylanase	(arabino)-xylan	1.278865

560

Figure captions

Fig. 1. Comparison of functional distribution of plant biomass degrading CAZy genes/enzymes expressed/produced by *A. niger* during cultivation on guar gum and related carbon sources. (A) Functional distribution of CAZyme encoding genes identified in the fungal transcriptome from guar gum cultures. (B) Distribution of identified proteins in *A. niger* exoproteomes on guar gum and mannan. The corresponding CAZy transcripts and proteins related to plant biomass degradation are divided by their predicted substrates. The pie chart represents the total number of genes or proteins identified according to its putative substrate. GH (glycoside hydrolase), AA (auxiliary activity), CE (carbohydrate esterase) and PL (polysaccharide lyase).

Fig. 2. Hierarchical clustering comparing the expression pattern of *A. niger* CAZyme encoding genes (A) after 2 h growth on D-glucose, D-mannose, D-galactose, mannan and guar gum, and (B) over 48 h of growth on guar gum. The transcriptional expression profiles from D-glucose, D-mannose, D-galactose, mannan and guar gum were normalized and clustered. The resulting heat maps were divided into clusters. The top color bar represent the gene expression value from downregulated (blue) to upregulated genes (yellow). Further details of the clusters are presented in Table S1, as are the description of the enzyme functions represented by the 3-letter codes.

Fig. 3. Venn diagrams showing the shared upregulated *A. niger* CAZy genes in (A) 2 h on guar gum (GG2h) vs. 2 h on D-glucose (Glc2h), D-mannose (Man2h), D-galactose (Gal2h), and mannan (Mnn2h), respectively, and (B) genes upregulated over 48 h on guar gum. A list containing the IDs and the respective annotation of the significantly upregulated ($p<0.01$) genes grouped in the Venn diagram is provided in Table S1.

Fig. 4. Relative abundance of CAZymes identified in *A. niger* exoproteomes after 24 h and 48 h of growth on guar gum (GG) and mannan (Mnn). The extracellular CAZymes were grouped by their predicted function towards (arabino)xylan, cellulose, galactomannan, inulin, pectin, xyloglucan, starch and not specific substrate. Difference in the percentage of relative abundances is represented by five sizes of circles from

589 0.01-0.09%, 0.1-0.9%, 1.0-3.9%, 4.0-7.8% and 12-15.7%. The different colors of the circles represent the
590 treatments of the samples: red = GG24h, green = GG48h, blue = Mnn24h and purple = Mnn48h. Details of
591 the enzymes and values are given in Table S2.
592
593 **Fig. 5.** Extracellular CAZyme activities detected from the culture supernatants of *A. niger* at 8 h, 24 h and
594 48 h of cultivation on guar gum and mannan. The activities of α-galactosidase (AGL), β-galactosidase
595 (LAC), endomannanase (MAN), β-mannosidase (MND), α-xylosidase (AXL), β-xylosidase (BXL), β-
596 glucosidase (BGL), cellobiohydrolase (CBH), glucoamylase (GLA), rhamnosidase (RHA), and
597 arabinofuranosidase (ABF) were evaluated. The vertical bars show the standard deviation of two biological
598 replicates and three technical replicate reactions.
599